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L41: Entry 31 of 37 File: USPT Aug 4, 1998

DOCUMENT-IDENTIFIER: US 5789245 A
TITLE: Alphavirus structural protein expression cassettes

BSPR:
In particular embodiments, the vectors described above include a viral junction region consisting of the nucleotide sequence as shown in FIG. 3, from nucleotide number 7579, to nucleotide number 7597 (SEQ. ID NO. 1). In alternative embodiments, where the vector includes a second viral junction, an E3 adenovirus gene may be located downstream from the second viral junction region. Vectors of the present invention may also further comprise a non-alphavirus (for example retrovirus, coronavirus, hepatitis B virus) packaging sequence located between the first viral junction region and the second viral junction region, or in the nonstructural protein coding region.

BSPR:
In still a further aspect, the present invention provides an alphavirus structural protein expression cassette, comprising a promoter and one or more alphavirus structural protein genes, the promoter being capable of directing the expression of alphavirus structural proteins. In various embodiments, the expression cassette is capable of expressing alphavirus structural proteins, such as an alphavirus structural protein selected from the group consisting of C, 6K, E3, E2, and E1.

BSPR:
Within another aspect, HBV-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an HBV-derived packaging cell line is provided, comprising an expression cassette which directs the expression of HBV core, preS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

BSPR:
Within other aspects of the present invention, methods are provided for delivering a heterologous nucleic acid sequence to an animal comprising the steps of administering to the warm-blooded animal a eukaryotic layered vector initiation system as described above. Within certain embodiments, the eukaryotic layered vector initiation system may be introduced into the target cells directly as a DNA molecule by physical means, as a complex with various liposome formulations, or as a DNA-ligand complex including the vector molecule (e.g., along with a polycation compound such as polylysine, a receptor specific ligand, or a psoralen inactivated virus such as Sendai or Adenovirus).

DEPR:
"Expression cassette" refers to a recombinantly produced nucleic acid molecule which is capable of directing the expression of one or more proteins. The expression cassette must include a promoter capable of directing the expression of said proteins, and a sequence encoding one or more proteins, said proteins preferably comprising alphavirus structural protein(s). Optionally, the expression cassette may include transcription termination, splice recognition, and polyadenylation addition sites. Preferred promoters include the CMV, MMTV, MoMLV, and adenovirus VA1RNA promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, hygromycin, phleomycin, histidinol, and DHFR.

DEPR:
"Recombinant alphavirus particle" refers to a capsid which contains an alphavirus vector construct. Preferably, the capsid is an alphavirus capsid and is contained

within a lipid bilayer, such as a cell membrane, in which viral-encoded proteins are embedded. In some instances, the alphavirus vector construct may be contained in a capsid derived from viruses other than alphaviruses (for example, retroviruses, coronaviruses, and hepatitis B virus). A variety of alphavirus vectors may be contained within the recombinant alphavirus particle, including the alphavirus vector constructs of the present invention.

DEPR:
3. THE ADENOVIRUS E3 GENE

DEPR:
Within another aspect of the invention, an adenovirus E3 gene is inserted into a tandem vector construct following the second viral junction region, in order to down-regulate HLA expression in alphavirus infected cells. Briefly, within various embodiments of the invention, repeated inoculations of a gene therapeutic into the same individual is desirable. However, repeated inoculations of alphaviruses such as the Sindbis virus may lead to the development of specific antibodies or cell-mediated immune response against Sindbis viral nonstructural proteins (NSPs). Thus, it may be necessary to mitigate the host immune response targeted to vector-specific proteins in order to administer repeated doses to the same individual.

DEPR:
Therefore, within one embodiment of the invention, products of the Adenovirus type 2 early region gene 3 are utilized in order to down-regulate the expression of integral histocompatibility antigens expressed on the surface of infected cells. Briefly, the E3 19,000 dalton (E3/19K) protein binds to, and forms a molecular complex with, class I H-2/HLA antigens in the endoplasmic reticulum, preventing terminal glycosylation pathways necessary for the full maturation and subsequent transport of the class I H-2/HLA antigens to the cell membrane. In target cells infected with an alphavirus vector encoding the Ad 2 E3 protein, co-expression of the viral nonstructural proteins in the context of class I antigens will not occur. Thus, it is possible to administer repeated doses of an alphavirus vector which expresses the Ad 2 E3 protein as a component of its therapeutic palliative to the same individual. A representative example of the use of the Adenovirus E3 gene is set forth in more detail below in Example 4A.

DEPR:
Within another aspect of the invention, a packaging sequence derived from a virus other than an alphavirus (for example, retrovirus, coronavirus, hepatitis B virus) is inserted into a tandem vector and positioned between the first (inactivated) viral junction region and the second, modified viral junction region. Briefly, nonalphavirus packaging sequences signal the packaging of an RNA genome into a virus particle corresponding to the source of the packaging sequence. For example, and as described in more detail below, a retroviral packaging sequence may be utilized in order to package an alphavirus vector into a retroviral particle using a retroviral packaging cell line. This is performed in order to increase the efficiency of alphavirus vector transfer into an alphavirus packaging cell line, or to alter the cell or tissue tropism of the alphavirus vector.

DEPR:
Therefore, within one aspect of the present invention DNA-based vectors (referred to as "Eukaryotic Layered Vector Initiation Systems") are provided which are capable of directing the synthesis of viral RNA in vivo. In particular, eukaryotic layered vector initiation systems are provided comprising a promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a construct which is capable of autonomous replication in a cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription termination. Briefly, such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. The first layer initiates transcription of the second layer, and comprises a promoter which is capable of initiating the 5' synthesis of RNA from cDNA (e.g., a 5' promoter), a 3' transcription termination site, as well as one or more splice sites and/or a polyadenylation site, if desired. Representative promoters suitable for use within the present invention include both eukaryotic (e.g., pol I, II, or III) and prokaryotic promoters, and inducible or non-inducible (i.e., constitutive) promoters, such as, for example, Murine Leukemia virus promoters (e.g., MoMLV), metallothionein promoters, the glucocorticoid promoter, Drosophila actin 5C distal promoter, SV 40 promoter, heat

shock protein 65 promoter, heat shock protein 70 promoter, immunoglobulin promoters, Mouse polyoma virus promoter ("Py"), rous sarcoma virus ("RSV"), BK virus and JC virus promoters, MMTV promoter, alphavirus junction region, CMV promoter, Adenovirus VA1RNA, rRNA promoter, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, and the lac promoter. The second layer comprises a vector construct which is capable of expressing one or more heterologous nucleotide sequences and of replication in a cell, either autonomously or in response to one or more factors. Within one embodiment of the invention, the second layer construct may be an alphavirus vector construct as described above.

DEPR:
A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., Nature 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, Biotechniques 6:616-627, 1988; Rosenfeld et al., Science 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., J. Vir. 63:3822-3828, 1989; Mendelson et al., Virol. 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, Adv. Exp. Med. Biol. 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, J. Virol. 65:532-536, 1991).

DEPR:
Within certain aspects of the present invention, methods are provided for delivering a heterologous nucleotide sequence to a warm-blooded animal, comprising the step of administering a eukaryotic layered vector initiation system as described above, to a warm-blooded animal. Eukaryotic layered vector initiation systems may be administered to warm-blooded animals either directly (e.g., intravenously, intramuscularly, intraperitoneally, subcutaneously, orally, rectally, intraocularly, intranasally), or by various physical methods such as lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7417, 1989), direct DNA injection (Acsadi et al., Nature 352:815-818, 1991); microprojectile bombardment (Williams et al., PNAS 88:2726-2730, 1991); liposomes of several types (see, e.g., Wang et al., PNAS 84:7851-7855, 1987); CaPO.sub.4 (Dubensky et al., PNAS 81:7529-7533, 1984); DNA ligand (Wu et al, J. of Biol. Chem. 264:16985-16987, 1989); administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al., Hum. Gene Ther. 3:147-154, 1992); via polycation compounds such as polylysine, utilizing receptor specific ligands; as well as with psoralen inactivated viruses such as Sendai or Adenovirus. In addition, the eukaryotic layered vector initiation systems may either be administered directly (i.e., in vivo), or to cells which have been removed (ex vivo), and subsequently returned.

DEPR:
Briefly, specific down-regulation of inappropriate or unwanted immune responses, such as in chronic hepatitis or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products which suppress surface expression of transplantation (MHC) antigen. Group C adenoviruses Ad2 and Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3 region of the virus. This gp 19 molecule binds to class I MHC molecules in the endoplasmic reticulum of cells, and prevents terminal glycosylation and translocation of class I MHC to the cell surface. For example, prior to bone marrow transplantation, donor bone marrow cells may be infected with gp 19-encoding vector constructs which, upon expression of the gp 19, inhibit the surface expression of MHC class I transplantation antigens. These donor cells may be transplanted with low risk of graft rejection and may require a minimal immunosuppressive regimen for the transplant patient. This may allow an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including lupus erythromiatis, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

DEPR:
The SINDBIS basic vector and SINDBIS-luciferase constructs described in sections A and B of Example 3, above, are inserted into the pVGELVIS vector configurations described previously in Example 2 such that expression of the heterologous gene from Sindbis vectors occurs after direct introduction of the plasmid DNA into cells. As described in Example 2, the ability to transfect alphavirus-based vector plasmid DNA directly onto cells resulting in expression levels of heterologous genes typical of transfection of RNA-based alphavirus vectors, without a primary step consisting of in vitro transcription of linearized template vector DNA, enhances greatly the utility and efficiency of certain embodiments of the alphavirus-based expression vector system. FIG. 8 is a schematic representation of one mechanism of expression of heterologous genes from a plasmid DNA alphavirus expression (ELVIS) vectors. Primary transcription in the nucleus and transport of the vector RNA to the cytoplasm leads to the synthesis of alphavirus nonstructural proteins which catalyze the expansion of heterologous gene mRNA via an antigenome intermediate which in turn serves as the template for production of genomic and subgenomic mRNA. The ELVIS vectors may be introduced into the target cells directly by physical means as a DNA molecule, as a complex with various liposome formulations, or as a DNA ligand complex including the alphavirus DNA vector molecule, a polycation compound such as polylysine, a receptor specific ligand, and, optionally, a psoralen inactivated virus such as Sendai or Adenovirus.

DEPR:
In accordance with the non-parenteral administration the present invention, the gene delivery vehicles, particularly those comprised of unencapsidated nucleic acid, may be complexed with a polycationic molecule to provide polycation-assisted non-parenteral administration. Such a method of gene delivery facilitates delivery of a gene via mediation by a physical particle comprised of multiple components that augment the efficiency and specificity of the gene transfer. In particular, polycationic molecules, such as polylysine and histone, have been shown to neutralize the negative charges on a nucleic acid molecule and to condense the molecule into a compact form. This form of molecule is transferred with high efficiency in cells, apparently through the endocytic pathway. The uptake in expression of the nucleic acid molecule in the host cell results after a series of steps, as follows: (1) attachment to cell surface; (2) cell entry via endocytosis or other mechanisms; (3) cytoplasmic compartment entry following endosome release; (4) nuclear transport; and (5) expression of the nucleic acid molecule carried by the gene delivery vehicle. In a further preferred embodiment, multi-layer technologies are applied to the polycation-nucleic acid molecule complex to facilitate completion of one or more of these steps. For example, a ligand such as asialoglycoprotein, transferrin, and immunoglobulin may be added to the complex to facilitate binding of the cell complex to the cell surface, an endosomal disruption component (e.g., a viral protein, a fusogenic peptide such as the n-terminus of the influenza virus hemaglutinin or an inactivated virus) is added to facilitate the release of DNA from the endosome, or a nuclear protein (or a peptide containing a nuclear localization signal) is added to facilitate the transport of the DNA into the nucleus. In a further preferred embodiment, the composition comprising the complex includes inactivated adenovirus particles (Curiel, D. T., et al., PNAS 88:8850-8854, 1991; Cristiano, R. J., PNAS 90:2122-2126 1993; Cotten, M., et al., PNAS 89:6094-6098 1992; Lozier, J. N., et al., Human Gene Therapy 5:313-322, 1994; Curiel, D. T., et al., Human Gene Therapy 3:147-154, 1992; Plank, C. et al., Bioconjugate Chem. 3:533-539, 1992; Wagner, E. et al., PNAS 88:4255-4259, 1991). The assorted components comprising the multi-layer complex may be varied as desired, so that the specificity of the complex for a given tissue, or the gene expressed from the gene delivery vehicle, may be varied to better suit a particular disease or condition.

DEPR:
More specifically, sequences which encode immunoreactive polypeptides of the pathogenic agents may, in certain embodiments, be chosen from a group that includes the Bunyaviridae (e.g., Rift Valley Fever virus (Giorgi et al., Virology 180:738-753, 1991; Collett et al., Virology 144:228-245, 1985)), Paramyxoviridae (e.g., Newcastle disease virus (Millar et al., J. Gen. Virol. 69:613-620, 1988; Chambers et al., Nucl. Acid. Res. 14:9051-9061, 1986; Schaper et al., Virology 165:291-295, 1988), and canine distemper virus (Curran et al., J. Gen. Virol. 72:443-447, 1991; Barrett et al., Virus Res. 8:373-386, 1987; Bellini et al., J. Virol. 58:408-416, 1986)), Togaviridae (e.g., WEE virus (Weaver et al., Virology 197:375-390, 1993), EEE virus (Chang et al., J. Gen. Virol. 68:2129-2142, 1987), and VEE virus (Kinney et al., Virology 152:400-413, 1986)), Rhabdoviridae (e.g.,

vesicular stomatitis virus (Gill et al., Virology 150:308-312, 1986; Gallione et al., J. Virol. 46:162-169, 1983; Banerjee et al., Virology 137:432-438, 1984), and rabies virus (Tordo et al., Nucl. Acid. Res. 14:2671-2683, 1986; Hiramatsu et al., Virus Genes 7:83-88, 1993; Kieny et al., Nature 312:163-166, 1984)), Coronaviridae (e.g., transmissable gastroenteritis virus (Britton et al., Molec. Micro. 2:89-99, 1988; Godet et al., Virology 188:166-175, 1992; Jackwood et al., Adv. Exp. Med. and Biol. 342:43-48, 1993), and feline infectious peritonitis virus (Reed et al., Adv. Exp. Med. and Biol. 342:17-21, 1993)), Reoviridae (e.g., porcine rotavirus (Burke et al., J. Gen. Virol. 75:2205-2212, 1994; Nishikawa et al., Nucl. Acid. Res. 16:11847, 1988)), Orthomyxoviridae (e.g. equine influenza (Gibson et al., Virus Res. 22:93-106, 1992; Dale et al., Virology 155:460-468, 1986)), Picornaviridae (e.g., FMD virus (Graham et al., Virology 176:524-530, 1990; Brown et al., Gene 75:225-233, 1989; Fross et al., Nucl. Acid. Res. 12:6587-6601, 1984)), and Herpesviridae (e.g., equine herpesvirus (Crabb et al., J. Gen. Virol. 72:2075-2082)).

DEPR:
In order to inhibit the host CTL response directed against viral specific proteins expressed in vector infected cells, in applications where repeated administration of the therapeutic is desired, the Adenovirus type 2 (Ad 2) E3/19K gene ATCC No. VR-846 is cloned into the pKSSINdlJRsjrc plasmid, immediately downstream from the junction region core. Briefly, Ad 2 is propagated in a permissive cell line, for example HeLa or Vero cells, and after evidence of cytopathologic effects, virions are purified from the cell lysate, and the Ad 2 DNA is purified from the virus.

DEPR:
Other gene products, in addition to the bcl-2 oncogene, which suppress apoptosis may likewise be expressed in an alphavirus packaging or producer cell line. Three viral genes which are particularly preferred include: the adenovirus EIB gene encoding the 19-kD protein (Rao et al., PNAS 89:7742-7746, 1992), the herpes simplex virus type 1 .sub.1 34.5 gene (Chou and Roizman, PNAS 89:3266-3270, 1992), and the AcMNPV baculovirus p35 gene (Clem et al., Science 254:1388-1390, 1991). These individual genes may be inserted into any commercially available plasmid expression vectors, under the control of appropriate constitutive eukaryotic transcriptional promoters, and also containing a selectable marker, using standard techniques. The expression vector constructs are subsequently transfected into cell lines as described above, and the appropriate selection is applied. Selection for stable integration of these genes and constitutive expression their products should allow for more extended vector production in cell lines found to be susceptible to alphavirus-induced apoptotic events. In addition, it is feasible that each gene product inhibits apoptosis by its own unique mechanism. Therefore, the genes may also be introduced into packaging or producer cell lines in various combinations in order to obtain a stronger suppressive effect. Finally, other gene products having similar effects on apoptosis can also be readily incorporated into packaging cell lines as they are identified.

DEPR:
In the derivation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin A, p33.sup.cdk2 and p34.sup.cdc2. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, src, and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., Cell 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with

cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., Cancer Research 54:3131-3135,1994).

DEPR:
The Sindbis E2 glycoprotein is synthesized as a precursor, PE2. This PE2 precursor along with the second viral glycoprotein, E1, associate in the endoplasmic reticulum and are processed and transported to the infected cell membrane as a heterodimer for virion incorporation. At some point during this processing, PE2 is cleaved into E3 and the mature virion glycoprotein E2. E3 is the 64 amino-terminal residues of PE2 and is lost in the extracellular void during maturation. The larger cleavage product, E2, is associated with E1 and anchored in what becomes the viral envelope. Host cell protease(s) is responsible for processing of the PE2 precursor, cleaving at a site that immediately follows a highly conserved canonical four amino acid (aa) residue motif, basic-X-basic-basic aa's. A mutant cell line derived from the CHO-K1 strain, designated RPE.40 (Watson et al., J. Virol 65:2332-2339, 1991), is defective in the production of Sindbis virus strain AR339, through its inability to process the PE2 precursor into the E3 and mature E2 forms. The envelopes of Sindbis virions produced in the RPE.40 cell line therefore contain a PE2/E1 heterodimer. RPE.40 cells are at least 100-fold more resistant to Sindbis virus infection than the parental CHO-K1 cells, suggesting an inefficiency in the ability of PE2 containing virions to infect these cells. The defective virions produced by the RPE.40 cell line can be converted into a fully infectious form by treatment with trypsin.

DEPR:
The development of alphavirus packaging cell lines is dependent on the ability to synthesize high intracellular levels of the necessary structural proteins: capsid, pE2 and/or E2, and E1. Unfortunately, high level expression of these proteins, in particular, the envelope glycoproteins E2 and E1, may lead to concomitant cytopathology and eventual cell death. Therefore structural protein expression cassettes have been designed with inducible regulatory elements which control the levels of gene expression, in addition to others which maintain constitutive levels of expression.

DEPR:
Variations of the lac operon-Sindbis structural protein gene expression cassettes also can be constructed using other viral, cellular or insect-based promoters. Using common molecular biology techniques known in the art, the lac operon and the RSV LTR promoter, or just the RSV LTR promoter, sequences can be switched out of the Stratagene pOP13 and pOPRSV1 vectors and replaced by other promoter sequences, such as the cytomegalovirus major immediate promoter (pOPCMV-SINSP); the adenovirus major late promoter (pOPAMLP-SINSP); the SV40 promoter (pOPSV-SINSP): or insect promoter sequences, which include the Drosophila metallothionein inducible promoter (pMET-SINSP), Drosophila actin 5C distal promoter (pOPA5C-SINSP), heat shock promoters HSP65 or HSP70 (pHSP-SINSP), or the baculovirus polyhedrin promoter (pPHED-SINSP).

DEPR:
Packaging cell lines may also be generated which segregate the integration and expression of the structural protein genes, allowing for their transcription as non-overlapping, independent RNA molecules. For example, the expression of capsid protein independently of glycoproteins E2 and E1, or each of the three proteins independent of each other, eliminates the possibility of recombination with vector RNA and subsequent generation of contaminating wild-type virus.

DEPR:
The glycoprotein genes, E1 and E2, are expressed together using one of the inducible systems previously described. For example, the Sindbis E1 and E2 genes are amplified from plasmid pVGSP6GEN using a primer pair complementary to Sindbis nucleotides 8440-8459 (forward primer) and Sindbis nts 11,384-11,364 (reverse primer). PCR amplification is performed using a standard three-temperature cycling protocol and the following oligonucleotide pair:

DEPR:
Alternatively, the E1 and E2 glycoproteins are expressed under the control of the replicon-inducible junction region promoter, described previously. The ELVIS expression plasmid pVGELVIS0SINBV-linker (Example 3) is digested with the enzyme Not I, and treated with calf intestinal alkaline phosphatase. PCR amplified Sindbis E1 and E2 glycoprotein genes digested with Not I (previous paragraph) are

then ligated to the ELVIS vector to generate a construct designated pVGELVIS-E1/E2. Plasmid pVGELVIS-E1/E2 subsequently is digested with the enzyme Bsp EI, removing most of the nonstructural protein gene coding region, and the remaining E1- and E2-containing vector DNA is re-ligated to itself, creating an inducible expression cassette identified as pVGELVdl-E1/E2. This glycoprotein expression vector is used to transfect cells that have been previously transfected with a capsid protein expression construct, and stable glycoprotein gene transfectants are identified by selection for G418 resistance. For both the capsid and envelope glycoprotein expression cassettes, additional mammalian or non-mammalian (including insect)-derived promoters, which may or may not be inducible, are readily substituted for those described above, using standard techniques known in the art.

DEPR:
Various alternative systems can be used to produce recombinant alphavirus particles carrying the vector construct. Each of these systems takes advantage of the fact that baculovirus, and the mammalian viruses vaccinia and adenovirus, among others, have been adapted recently to make large amounts of any given protein for which the gene has been cloned. (Smith et al., Mol. Cell. Biol. 3:12, 1983; Piccini et al., Meth. Enzymology 153:545, 1987; and Mansour et al., Proc. Natl. Acad. Sci. USA 82:1359, 1985). These and other viral vectors are used to produce proteins in tissue culture cells by insertion of appropriate genes into the viral vector and can be readily adapted to make alphavirus vector particles.

DEPR:
For example, adenovirus vectors are derived from nuclear replicating viruses and can be modified so they are defective. Heterologous genes are inserted into these vectors either by in vitro construction (Ballay et al., EMBO J. 4:3861, 1985) or by recombination in cells (Thummel et al., J. Mol. Appl. Genetics 1:435, 1982), and used to express proteins in mammalian cells. One preferred method is to construct plasmids using the adenovirus major late promoter (MLP) driving: (1) alphavirus structural proteins; and (2) an alphavirus vector construct. The alphavirus vector in this configuration still contains a modified junction region, and would allow the transcribed RNA vector to be self-replicating, as in previously described configurations.

DEPR:
These plasmids are then used to make adenovirus genomes in vitro (Ballay et al., EMBO. J. 4:3861, 1985). The recombinant adenoviral genomes, which are replication defective, are separately transfected into 293 cells (ATCC #CRL 1573, a human cell line making adenovirus E1A protein), to yield pure stocks of defective adenovirus vectors expressing either alphavirus structural proteins or alphavirus vectors. Since the titres of such vectors are typically 10.sup.7 -10.sup.11 /ml, these stocks are then used to infect tissue culture cells simultaneously at high multiplicity of infection, resulting in the production of alphavirus proteins and vector genomes at high levels. Since the adenovirus vectors are defective, little or no direct cell lysis will occur and vectors are harvested from the cell supernatants. Similar approaches are readily carried out using recombinant vaccinia virus vectors constructed by inserting the alphavirus sequences into the shuttle plasmid pK (Bergmann et al., Eur. J. Immunol. 23:2777, 1993) for in vivo recombination into the vaccinia WR strain.

DEPR:
The tissue and cell-type specificity of alphaviruses is determined primarily by the virus-encoded envelope proteins, E1 and E2. These virion structural proteins are transmembrane glycoproteins embedded in a host cell-derived lipid envelope that is obtained when the viral particle buds from the surface of the infected cell. The envelope surrounds an icosahedral nucleocapsid, comprised of genomic RNA complexed with multiple, highly ordered copies of a single capsid protein. The E1 and E2 envelope glycoproteins are complexed as heterodimers which have been reported to assemble into trimeric structures, forming the characteristic "spikes" on the virion surface. In addition, the cytoplasmic tails of these proteins interact with the nucleocapsids, initiating the assembly of new viral particles (Virology 193:424, 1993). Properties ascribed to the individual glycoproteins of Sindbis virus include receptor binding by glycoprotein E2 (Virology 181:694, 1991) and glycoprotein E1-mediated fusion of the virion envelope and the endosomal membrane, resulting in delivery of the nucleocapsid particle into the cytoplasm (New Aspects of Positive-Stranded RNA Virus, pp. 166-172, 1990).

DEPR:
The present invention recognizes that by disrupting glycoprotein activity (in particular, but not limited to that of E2) and co-expressing an intact heterologous glycoprotein, or by creating hybrid envelope gene products (i.e., specifically, an alphavirus envelope glycoprotein having its natural cytoplasmic domain and membrane-spanning domain, with its exogenous binding domain replaced by the corresponding domain(s) from a different envelope glycoprotein, or by replacing the E2 and/or E1 glycoproteins with those of other alphaviruses or their derivatives which differ from that of the vector in their tissue tropism, the host range specificity may be altered without disrupting the cytoplasmic functions required for virion assembly. Alternatively, by replacing one or more of the alphavirus structural proteins with the structural protein(s) of another virus and introducing the corresponding viral packaging sequence into the alphavirus vector construct, assembly of recombinant alphavirus vector constructs into particles of other virus types can be achieved. Thus, recombinant alphavirus particles can be produced which have an increased affinity for pre-selected target cells, depending on the tropism of the protein molecule(s) or domain(s) introduced.

DEPR:
In one embodiment, substitution of the analagous envelope glycoproteins E1 and/or E2 from other alphaviruses or their variants is performed to alter tissue tropism. For example, Venezuelan equine encephalitis virus (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector constructs packaged in a cell line expressing the VEE structural proteins display the same lymphotropic properties as the parental VEE virus from which the packaging cell structural protein gene cassette was obtained.

DEPR:
Similarly, the packaging signal from a coronavirus can be incorporated into the alphavirus vector. For example, the 190 nt packaging signal from mouse hepatitis virus (MHV), comprising nts 2899 to 3089 (Fosmire et al., J. Virol. 66:3522, 1992), is amplified in a standard three cycle PCR protocol using THERMALASE.TM. polymerase, DIssF plasmid MP51-2 (Fosmire et al., J. Virol. 66:3522, 1992) as the template, and the following oligonucleotides, which contain flanking ApaI recognition sites:

DEPR:
In each case, the packaging sequences from HBV, coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the altenative approaches described in Example 8.

DEPR:
On the other hand, it may be desired to administer repeated doses to an individual; thus the antisense and hairpin palliative would be inserted downstream of the adenovirus E3 or human cytomegalovirus H301 genes, which down-regulate the expression of MHC class I molecules in infected cells. Insertion of the antisense and hairpin palliatives is accomplished in the vectors from Examples 3 and 4 shown below, between the Cla I and Xba I sites:

DEPR:
Further, in some applications, for example systemic expression of protein, multiple dose administration to an individual is required. In these applications, prolonged expression of the therapeutic palliative without induction of an immune response targeted towards the vector infected cell is desired. In this configuration, the IFN-.alpha.HRBZ moiety could be inserted upstream of the adenovirus E3 or human cytomegalovirus H301 genes, which down-regulate the expression of MHC class I molecules in infected cells. Following the gene which modulates MHC class I expression is, consecutively, an IRES element selected from among the group described in Example 5, and the therapeutic palliative. Ordered insertion of the hairpin ribozyme, Ad E3 or CMV H301, IRES, and heterologous gene of interest components along the multiple cloning sequence located in the vector

between the vector junction region and 3' end is accomplished by modification with the appropriate restriction enzyme recognition sites of the component 5' and 3' ends. In these constructions, functional INF-a hairpin ribozyme palliatives will be present at the level of both subgenomic and positive stranded genomic Sindbis vector RNA.

DEPR:
Any of the above-described vector constructs may be utilized along with packaging cell lines described in Example 7, in order to produce recombinant alphavirus particles suitable for administration to humans or animals (either directly or indirectly), or for infecting target cells. Such vector constructs may also introduced directly into target cells as a "naked" DNA molecule, as a DNA complex with various liposome formulations, or as a DNA ligand complex including the alphavirus DNA vector molecule (e.g., along with a polycation compound such as polylysine, a receptor specific ligand, or a psoralen inactivated virus such as Sendai or Adenovirus).

DEPR:
In accordance with the non-parenteral administration the present invention, the gene delivery vehicles, particularly those comprised of unencapsidated nucleic acid, may be complexed with a polycationic molecule to provide polycation-assisted non-parenteral administration. Such a method of gene delivery facilitates delivery of a gene via mediation by a physical particle comprised of multiple components that augment the efficiency and specificity of the gene transfer. In particular, polycationic molecules, such as polylysine and histone, have been shown to neutralize the negative charges on a nucleic acid molecule and to condense the molecule into a compact form. This form of molecule is transferred with high efficiency in cells, apparently through the endocytic pathway. The uptake in expression of the nucleic acid molecule in the host cell results after a series of steps, as follows: (1) attachment to cell surface; (2) cell entry via endocytosis or other mechanisms; (3) cytoplasmic compartment entry following endosome release; (4) nuclear transport; and (5) expression of the nucleic acid molecule carried by the gene delivery vehicle. In a further preferred embodiment, multi-layer technologies are applied to the polycation-nucleic acid molecule complex to facilitate completion of one or more of these steps. For example, a ligand such as asialoglycoprotein, transferrin, and immunoglobulin may be added to the complex to facilitate binding of the cell complex to the cell surface, an endosomal disruption component (e.g., a viral protein, a fusogenic peptide such as the n-terminus of the influenza virus hemaglutinin or an inactivated virus) is added to facilitate the release of DNA from the endosome, or a nuclear protein (or a peptide containing a nuclear localization signal) is added to facilitate the transport of the DNA into the nucleus. In a further preferred embodiment, the composition comprising the complex includes inactivated adenovirus particles (Curiel, D. T., et al., PNAS 88:8850-8854, 1991; Cristiano, R. J., PNAS 90:2122-2126 1993; Cotten, M., et al., PNAS 89:6094-6098 1992; Lozier, J. N., et al., Human Gene Therapy 5:313-322, 1994; Curiel, D. T., et al., Human Gene Therapy 3:147-154, 1992; Plank, C. et al., Bioconjugate Chem. 3:533-539, 1992; Wagner, E. et al., PNAS 88:4255-4259, 1991). The assorted components comprising the multi-layer complex may be varied as desired, so that the specificity of the complex for a given tissue, or the gene expressed from the gene delivery vehicle, may be varied to better suit a particular disease or condition.

DEPL:
A. INSERTION OF ADENOVIRUS EARLY REGION E3 GENE INTO SINDBIS VECTORS

DEPL:
Following amplification, the PCR amplicon is digested with ApaI, purified from a 1.5% agarose gel using MERMAID.TM., and ligated into pKSSINdlJRsjrc, prepared as described for HBV. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., NAR 11:883, 1983); membrane (M protein, Armstrong et al., Nature 308:751, 1984); and spike (S protein, Luytjes et al., Virology 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVSdl-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Addtional coronaviruses, for example, human coronaviruses OC43 (ATCC

#VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

CLPR:
13. The expression cassette of any one of claims 1, 2, 3, 5, 6 or 7, wherein the alphavirus structural protein gene or glycoprotein gene encodes a protein selected from the group consisting of alphavirus structural proteins E3, E2 and E1.